IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No.	:	09/936,145
Applicants	:	Yasushi Inoue et al.
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Title	:	PROMOTERS
Group Art Unit	:	1652
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Commissioner for Patents
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DECLARATION UNDER 37 C.F.R. § 1.132

I, Yasushi Inoue, declare as follows:

1. I am one of the named inventors of the invention described and claimed in the above-identified application.

2. I am a citizen of Japan, and reside at c/o Showa Sangyo Co., Ltd., Sogokenkyujyo Baiokenkyusenta, 16, Sakura 1-chome, Tsukuba-shi, Ibaraki 305-0003 Japan. I studied food chemistry at the Department of Agriculture of Nagoya University and received a Master's degree in 1994 and received a Doctor's degree in Agriculture form Nagoya University in 2003. I am an employee and researcher at Showa Sangyo Co., Ltd., the assignee of the above-captioned application. Since 1994, I have been employed by Showa Sangyo Co., Ltd. and have been involved in the study of carbohydrate and enzymes in the Department of Research and Development.

I have read and am thoroughly familiar with the contents of the above-identified patent application as well as of the prior art references cited in the application. I have read and I understand amended claims 4 and 31.

3. As an expert in the field of molecular biology, I can attest that it is well known that not only is nucleotide sequence extremely important for promoter expression, but the length of the nucleotide sequence, or the number of bases, is extremely important as well.

4. There is much documentation in the art demonstrating that the conservation of promoter structure in *Bacillus subtilus*, which is related closely to *Bacillus amyloliquefaciens* of the present invention, is much stricter than what is observed generally, for example in *E. coli*. *B. subtilis* RNA polymerase demands high fidelity to the canonical −35 and −10 promoter hexanucleotides. Furthermore, the *B. subtilis* ribosome may require extensive complementarity to messenger RNA.

5. The present invention modifies the promoter of α-amylase derived from *B. amyloliquefaciens* by extending the Shine-Delgarno sequence by three nucleotides and by inserting at least one restriction site in the 3'end region. These novel modifications results in new and unexpected results. As illustrated in Table 1 on page 18 of the specification, in recombinant *B. subtilis* having an MPase gene, the productivity for MPase was increased over four-fold by using a promoter of α-amylase derived from *B. amyloliquefaciens* having at least one restriction site in the 3' end: MPase activity in units/l was 240,000 for the restriction enzyme sites BamHI and 240,000 for BamHI, SmaI, KpnI, SacI and EcoRI, whereas *B. subtilis* using a promoter of α-amylase derived from *B. amyloliquefaciens* without at least one restriction site in the 3' end, MPase activity in units/l was 50,000. In recombinant *B. subtilis* microorganisms having a TPase gene, the productivity for TPase was increased over four-fold by using a promoter of α-amylase derived from *B. amyloliquefaciens* having at least one restriction site in the 3' end: TPase activity in units/l was 300,000 for the restriction enzyme sites BamHI and 300,000 for BamHI, SmaI, KpnI, SacI and EcoRI, whereas *B. subtilis* using a promoter of α-amylase derived from *B. amyloliquefaciens* without at least one restriction site in the 3' end, MPase activity in units/l was 70,000.

6. I understand Palva et al. to disclose the full sequence of the promoter derived from *Bacillus amyloliquefaciens* in Fig. 2, third line from the top: 5'-G AGA GGG AGA

GGA AAC-3'. The sequence G AGA GGG AGA GGA from the 5' end, indicated by the solid and broken line in Fig. 2, constitutes a Shine-Dalgarno sequence, which is believed to be a potential RNA polymerase recognition site. The sequence 5'-G AGA GGG AGA GGA AAC-3' disclosed by Palva et al. contains the 10 nucleotides from the 3' end (underlined nucleotides) that are modified in the present invention. Based on the above requirement that the conservation of the promoter structure in *B. subtilus*, i.e., *B. amyloliquefaciens*, be preserved in order for optimal promoter expression, as well as the knowledge of the importance of sequence length on promoter expression, one would not be motivated to extend the Shine-Delgarno sequence by the three nucleotides, TCC, of the present invention. One would be more motivated to preserve such a sequence in order to preserve the original sequence of α-amylase DNA promoters so as to ensure optimal promoter activity.

7. It is my expert opinion that the new and unexpected enhanced expression of the α-amylase promoter derived from the microorganism *B. amyloliquefaciens* of the present invention is due directly to the novel nucleotide sequence, nucleotide length and position, i.e., 3' terminus, of the α-amylase promoter. Furthermore, Palva et al. and those skilled in the art neither teach nor suggest the specific modifications of nucleotide sequence, length and position of the α-amylase promoter in the region of the critical Shine-Delgarno sequence of the α-amylase promoter, which results in the above-described new and unexpected results of the present invention. Indeed, Palva et al. and common knowledge in the art actually teach away from the novel modifications of the α-amylase promoter of the present invention, based on the long believed dogma that the conservation of the promoter structure in *B. subtilis* be preserved for optimal promoter expression.

8. I declare further that all statements made herein of my own knowledge are true and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application and any patent issuing thereon.

Yasushi Inoue

Yasushi Inoue, Ph.D.
Date 18 March 2004